

**APPLICATION
FOR
UNITED STATES LETTERS PATENT**

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Title: INTERNAL IMAGE ANTIBODIES FOR OPTICAL
IMAGING AND THERAPY

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SPECIFICATION

INTERNAL IMAGE ANTIBODIES FOR OPTICAL IMAGING AND THERAPY

Field of the Invention

5 The invention relates generally to diagnosis and therapy using
photoactive compounds. More particularly, the invention relates to
bioconjugates comprising internal image antibodies or their fragments attached
to photoactive molecules.

Background of the Invention

10 The use of visible, near-infrared (NIR), and low-energy ultraviolet light
in clinical practice is growing rapidly. Compounds absorbing or emitting in the
visible, NIR, or long wavelength (UV-A, >350 nm) region of the electromagnetic
spectrum are potentially useful for diagnostic techniques such as tomographic
or planar imaging, endoscopic examination, optoacoustic imaging and
15 sonofluorescence imaging.

Notwithstanding the importance of photodiagnostic applications, a
major advantage of biomedical optics lies in its therapeutic potential.
Phototherapy has been demonstrated to be a safe and effective procedure for
the treatment of various surface lesions, both external and internal. Its efficacy

is comparable to radiotherapy, but without the harmful radiotoxicity to critical non-target organs.

The use of fluorescent dyes and covalent dye conjugates for the detection of various species in body fluids is well known in the field of *in-vitro* immunodiagnostics. Fluorescent dyes have also been used as chemosensors and in fluorescence microscopy. Dyes and dye-immunoconjugates have been widely used in immunohistology, and in immunochemical detection of various small and large biomolecules in body fluids using enzyme-linked immunosorbent assay (ELISA) procedures. More recently, indocyanine green, a highly fluorescent polyene dye, has been used for monitoring cardiac output, assessing hepatic function, and tomographic imaging of tumors.

Specific targeting of fluorescent dyes to a particular site, such as a tumor, has advantages over non-specific localization of these dyes in various tissues. A known method of targeting is by attaching the dye or other effector molecule to an antibody that binds at a target site. Antibodies can tolerate the attachment of a reasonable number of haptens, such as up to about five haptens, on their surfaces while still substantially retaining their binding properties. In contrast, conjugation of haptens to molecules that are similar in size, such as drugs or hormones, most often but not always, obviate the binding properties of the effector molecule to the receptor. This is due to the fact that the large size of antibodies permit the attachment of haptens away from the combining site of the antibodies.

We have demonstrated that indocyanine green dye covalently attached to octreotate retains the somatostatin receptor binding properties, as reported in Achilefu et al., Investigative Radiology, 2000, vol. 35, p. 479, which

is expressly incorporated by reference herein in its entirety. This observation, however, is not general and it is not possible at this time to predict *a priori* the binding properties of small molecule bioconjugates with a high degree of confidence. In contrast, the binding properties of antibody conjugates is generally predictable.

To target a receptor using antibodies, however, anti-receptor antibodies are required. Conventional methods of producing anti-receptor monoclonal antibodies require the isolation of pure receptors. This is often not possible for various reasons, including the lack of stability of many biological receptors. Thus, most receptor targeting with anti-receptor antibodies have been elusive.

The idiotypic network theory of Jerne (Immunological Reviews, 1984, 79, 5-24) proposes that the variable regions of antibodies (i.e. idiotypes) act as immunogens to give rise to a secondary set of antibodies, called anti-idiotypes. An anti-idiotypic antibody is an antibody raised against a first antibody.

The binding site of an antibody is the particular region of the antibody molecule which specifically binds to the recognized epitope. In particular, if antibodies are developed against a ligand that binds to a certain receptor within the body, then a subpopulation of the resulting anti-idiotypic population, which is referred to as 'internal images', may contain antibodies that will likewise bind to the same receptor, due to the sharing of a common epitope between the ligand and the internal image. Essentially, the anti-idiotypic antibody mimics the original ligand or drug that is specific to the particular biological receptor.

Application of the principles proposed by Jerne has led to the isolation of a number of internal image antibodies directed at various biological

receptors, without ever having to isolate and purify the natural receptor.

Examples of these receptors include receptors for thyroid stimulating hormone (TSH), glucocorticoid, and adenosine. Lue et al. (Proceedings of the National Academy of Sciences, 1994, 91, 10690-10694) have used this approach to study the mechanism of an anti-cancer compound, taxol. Thus, the use of internal image antibodies with photoactive molecules for photodiagnostic and phototherapeutic purposes is desirable.

Summary of the Invention

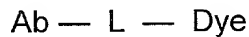
The composition and use of internal image antibody conjugates to achieve specificity for photodiagnostic and phototherapeutic applications, without the need to isolate a particular receptor, are disclosed. The invention discloses novel bioconjugates of internal image anti-idiotypic antibodies, or antibody fragments which contain the desired epitope for binding (hereinafter referred to as antibody fragment), that are attached to photoactive molecules (PM) for photodiagnostic or phototherapeutic purposes. These are generally represented by the following structure:



where Ab is a whole internal image antibody or antibody fragment directed to a particular biological receptor and PM is a photoactive molecule such as dyes, photosensitizers or precursors for producing reactive intermediates. The internal image antibody is directed to a biological receptor, for example, receptors for steroids, cardiac glycosides, somatostatin, bombesin, cholecystokinin (CCK), neurotensin, or heat sensitive bacterioendotoxin (ST). In some embodiments, a linker may link Ab and PM, represented as Ab-L-PM.

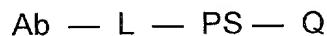
The linker may be -HNCONH-, -HNCSNH-, -HNCO-, -CONH-, -S(CH₂)_mCONH-, or -S-(N-succinimido)-(CH₂)_nCONH-, with m and n varying from 1 to 10.

In one embodiment, the invention discloses novel bioconjugates of internal image antibodies or antibody fragments attached to a photoactive dye for photodiagnostic purposes. In this embodiment, PM is a dye and the conjugate has the general formula:



where Ab is a whole or fragmented internal image antibody directed at a particular biological receptor and L is an optional linker. The dye may be a chromophore or a fluorophore useful for photodiagnostic purposes and may be derived from cyanines, indocyanines, phthalocyanines, rhodamines, phenoxazines, phenothiazines, phenoselenazines, fluoresceins, porphyrins, benzoporphyrins, squaraines, corrins, croconiums, azo compounds, methine dyes, and/or indolenium dyes.

In another embodiment, the invention discloses novel bioconjugates of internal image antibodies or antibody fragments attached to one or more photoactive molecules for phototherapeutic purposes. In this embodiment, PM is a photosensitizer (PS), which either by itself or associated with a precursor (Q), produces reactive intermediates. In this embodiment, the conjugate has the general formula:



where Ab is as previously described; L is optional as previously described; PS is a photosensitizing aromatic or a heteroaromatic radical derived from benzenes, polyfluorobenzenes, naphthalenes, naphthoquinones, anthracenes, anthraquinones, phenanthrenes, tetracenes, naphthacenediones, pyridines,

quinolines, isoquinolines, indoles, isoindoles, pyrroles, imidazoles, pyrazoles, pyrazines, purines, benzimidazoles, benzofurans, dibenzofurans, carbazoles, acridines, acridones, phenanthridines, thiophenes, benzothiophenes, dibenzothiophenes, xanthenes, xanthonenes, flavones, coumarins, and anthacylines; and Q is either a precursor for producing reactive intermediates such as free radicals, nitrenes, carbenes, and the like, or may be an azide (-N₃), a cyclic azo compound (-N=N-), or a sulfenate (-O-S-).

The invention also discloses a method for performing a diagnostic procedure using internal image antibody bioconjugates. In the method, a ligand is selected that binds to a biological receptor, such as the receptor for steroids, cardiac glycosides, somatostatin, bombesin, cholecystokinin, neurotensin, and heat sensitive bacterioendotoxin. The receptor-binding ligand may be one or more drugs, hormones, peptides, carbohydrates, nucleosides, peptidomimetics, glycomimetics, or biosynthetic intermediates. The ligand used as the first generation antigen may be the complete ligand, or may be a certain region of the ligand such a fragment that binds the receptor with high affinity, etc. The receptor-binding ligand is prepared as a first generation antigen, optionally conjugating the receptor-binding ligand to an immunogenic carrier. The immunogenic carrier may be, for example, bovine serum albumin, rabbit serum albumin, or human serum albumin.

First generation monoclonal antibodies are generated against the first generation antigen, and the specific monoclonal antibodies directed to the desired receptor-binding ligands are isolated. Monoclonal anti-idiotypic antibodies against the first generation antibodies are then generated, and internal image anti-receptor antibodies from the population of anti-idiotypic

antibodies are isolated. The internal image anti-receptor antibodies are then conjugated to a dye by conventional methods known to one skilled in the art.

In use, an effective amount of the internal image antibody conjugate is administered to a patient, and the conjugate is allowed to accumulate at the target site. The target site is then exposed to light having a wavelength in the range of 300 to 1200 nm, and having sufficient power and fluency to image the target tissue using, for example, absorbance, fluorescence, or scattering methods. The imaging can be obtained by a CCD camera or use optical tomographic equipment. All of these procedures are known to one skilled in the art.

The invention also discloses a method of performing a phototherapeutic procedure using internal image antibody bioconjugates. The internal image antibody and ligand are as previously described for use in a photodiagnostic method. Rather than a dye, however, a photosensitizer (PS) is conjugated to the internal image antibody and linker. The PS may be benzenes, polyfluorobenzenes, naphthalenes, naphthoquinones, anthracenes, anthraquinones, phenanthrenes, tetracenes, naphthacenediones, pyridines, quinolines, isoquinolines, indoles, isoindoles, pyrroles, imidiazoles, pyrazoles, pyrazines, purines, benzimidazoles, benzofurans, dibenzofurans, carbazoles, acridines, acridones, phenanthridines, thiophenes, benzothiophenes, dibenzothiophenes, xanthenes, xanthonenes, flavones, coumarins, and/or anthacylines. The PS may also be associated with a precursor (Q) which may be an azide ($-N_3$), a cyclo azo compound ($-N=N-$), or a sulfenate ($-O-S-$).

In use, an effective amount of the internal image antibody conjugate is administered to a patient, and the conjugate is allowed to accumulate at the

target site. The target site is exposed to light having a wavelength in the range of 300 to 1200 nm with sufficient power and fluency to treat the impaired tissue, for example, by inducing necrosis or apoptosis.

The invention will be further appreciated in light of the following
5 figures, detailed description, and examples.

Brief Description of the Drawings

FIG. 1 is a schematic pathway for the synthesis of a photodiagnostic conjugate.

FIG. 2 is a schematic pathway for the synthesis of a phototherapeutic
10 conjugate.

Detailed Description

The invention is directed to a diagnostic and/or therapeutic composition of an internal image antibody for a biological receptor (anti-receptor internal image antibody) that is conjugated to a photoactive molecule
15 or molecules. This composition has the general formula:



where Ab is a whole or fragmented internal image antibody directed at a particular biological receptor and PM is a photoactive molecule or molecules such as a dye or a photosensitizer which either by itself or with an attached
20 precursor produces reactive intermediates. A linker L may optionally be used to link Ab and PM, represented as Ab-L-PM.

For a diagnostic composition, the conjugate consists of an internal image antibody and a dye. For a therapeutic composition, the conjugate consists of an internal image antibody and a photosensitizer for producing
25 reactive intermediates. The internal-image antibody may be a whole antibody,

or may be antibody fragments that contain the desired epitope for binding which have been cleaved enzymatically or chemically to produce Fab, Fab', (Fab')₂, or other smaller fragments that retain antigen binding properties. The methods of cleaving antibodies to produce such fragments, and the uses of these

5 fragments, are known to one of skill in the art. Method for preparing antibody fragments are known to one skilled in the art, for example, by enzymatic digestion with papain or pepsin of the whole antibody, as described by Parham et al. (J. Immunol Methods, 1982, 53, 133-173) and Mariani et al. (Molecular Immunology, 1991, 1991, 69-77), the relevant sections of each being expressly
10 incorporated by reference herein.

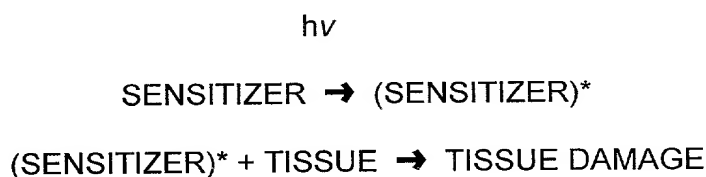
The invention is also directed to a method for performing a medical procedure by administering an effective amount of a composition of the anti-receptor internal image antibody conjugate. Photodiagnostic and/or phototherapeutic agents are targeted to the desired site using receptor-specific
15 internal image antibodies to receptors that expressed at these sites. The method may be used in various biomedical applications, such as in the diagnosis and/or therapy of some types of coronary disorders, certain types of tumors, certain types of lesions, etc. These included, but are not limited to, diagnosing and/or therapy of regions that are necrotic, ischemic, thrombic,
20 inflammatory, contain impaired vasculature, in which apoptosis is desirable, etc.

The use of internal image antibodies as carriers for targeted photodiagnostic and phototherapeutic applications is desirable, since it provides the benefit of high specificity without the problems associated with small molecule bioconjugates. The use of internal images in diagnostic and
25 therapeutic medicine was first proposed by us (Rajagopalan, Tissue specific

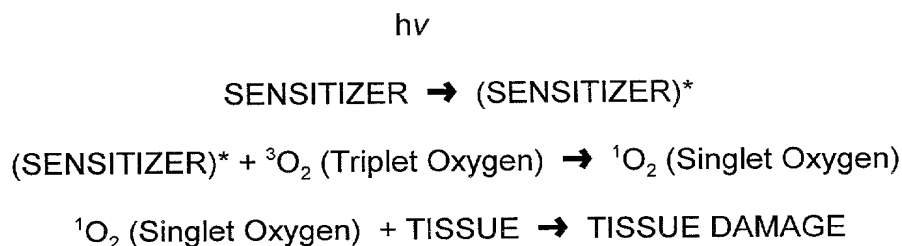
imaging agents using internal image anti-idiotypic antibodies, WO 93/00934).

The inventive approach disclosed herein of utilizing internal image antibodies for targeting specific receptors has several advantages. Purified receptors need not be used to develop the anti-receptor antibodies; this is advantageous since it is often difficult and sometimes impossible to isolate pure, stable receptors for immunization. The receptor binding affinity of the original ligand and the corresponding internal image antibody is very similar. The attachment of an effector molecule, such as a photoactive agent or a photosensitizer, to the large antibody molecule does not significantly perturb its receptor binding capability.

Photosensitizers operate via two distinct pathways, termed Types 1 and 2. Type 1 mechanisms are shown in the following scheme:



Type 1 mechanism involves direct energy or electron transfer from the photosensitizer to the cellular components thereby causing cell death. Type 2 process involves two distinct steps as shown in the following scheme:



In the first step, singlet oxygen is generated by energy transfer from the triplet excited state of the photosensitizer to the oxygen molecules surrounding the

tissues. In the second step, collision of a singlet oxygen with the tissues promotes tissue damage. In both Type 1 and Type 2 mechanism, the photoreaction proceeds via the lowest triplet state of the sensitizer. Hence, relatively long triplet lifetime is required for effective phototherapy whereas short triplet lifetime is necessary for photodiagnostics in order to avoid photo damage. The inventive method can be used in photodiagnostic or phototherapeutic applications operating by either the Type 1 or Type 2 mechanism, as described by Jori (*EPA Newsletter*, 1997, No. 60, 12-18), which is expressly incorporated by reference herein in its entirety.

Anti-idiotypic antibodies are made by developing antibodies against a first antibody that binds specifically to a certain ligand directed to a desired receptor within the body. The resulting anti-idiotypic antibody binds to the same receptor as the ligand, due to the topological similarity of the anti-idiotypic antibody and the ligand. The anti-idiotypic antibodies employed in the present invention are made according to well established hybridoma technology of Kohler and Milstein as is known to one of skill in the art and as modified by others (European Patent 0386275). As previously described, antibody fragments that retain the desired epitope for binding may be used. The whole or fragmented, natural or recombinant anti-idiotypic antibodies can be coupled to the photoactive molecule such as a chromophore, fluorophore, or photosensitizer by any one of the several methods known to one of ordinary skill in the art.

The inventive diagnostic compositions comprise anti-receptor internal image antibody conjugates of formula 1,



wherein Ab is a whole or fragmented internal image antibody which retain binding offinities directed at a particular biological receptor. The internal image antibodies of the invention are directed at receptors for steroids, cardiac glycosides (e.g., digoxin), somatostatin, bombesin, neurotensin, cholecystokinen (CCK) and heat sensitive bacterioendotoxin (ST), (see, for example, U.S. Patent No. 5,518,888, which is expressly incorporated by reference herein in its entirety). In some embodiments, a linker (L) links Ab and Dye, represented as Ab-L-Dye. The linker may be -HNCONH-, -HNCSNH-, -HNCO-, -CONH-, -S(CH₂)_mCONH-, and -S-(N-succinimido)-(CH₂)_nCONH-, where m and n vary from 1 to 10. The dye is a fluorophore or a chromophore capable of absorbing or emitting light having a wavelength in the range of 300-1200 nm.

In one particular embodiment of a diagnostic conjugate, Ab is directed at a receptor for steroids, cardiac glycosides, bombesin, neurotensin, or ST; L is a linker and may be -HNCONH-, -HNCSNH-, -HNCO-, or -S-(N-succinimido)-(CH₂)_nCONH- and n varies from 1 to 6; and the dye is a chromophore or fluorophore derived from a cyanine, an indocyanine, a phthalocyanine, a rhodamine, a phenoxazine, a phenothiazine, a phenoselenazine, a fluorescein, a porphyrin, a benzoporphyrin, a squaraine, a corrin, a croconium, an azo compound, a methine dye, an indolenium dye, and the like.

In another particular embodiment of a diagnostic conjugate, Ab is directed at a receptor for steroids, bombesin, neurotensin, or heat sensitive bacterioendotoxin; L is a linker and may be -HNCONH-, -HNCO-, or -S-(N-succinimido)-(CH₂)_nCONH- and n varies from 1 to 6; and the dye is an aromatic

or a heteroaromatic radical derived from a cyanine, an indocyanine, a rhodamine, a phenothiazine, a fluorescein, or an azo compound.

The inventive therapeutic compositions comprise anti-receptor internal image antibody conjugates of formula 2,



wherein Ab is a whole or fragmented internal image antibody directed at a particular biological receptor. The internal image antibodies of the invention are directed at receptors for steroids, cardiac glycosides (e.g., digoxin), somatostatin, bombesin, cholecystokinin, neurotensin, or heat sensitive bacterioendotoxin. A linker L may link Ab and PS or PS-Q, represented as Ab-L-PS(-Q), and L may be -HNCONH-, -HNCSNH-, -HNCO-, -CONH-, -S(CH₂)_mCONH-, or -S-(N-succinimido)-(CH₂)_nCONH-, where m and n vary from 1 to 10. PS is a photosensitizing aromatic or a heteroaromatic radical derived from a benzene, a polyfluorobenzene, a naphthalene, a naphthoquinone, an anthracene, an anthraquinone, a phenanthrene, a tetracene, a naphthacenedione, a pyridine, a quinoline, an isoquinoline, an indole, an isoindole, a pyrrole, an imidazole, a pyrazole, a pyrazine, a purine, a benzimidazole, a benzofuran, a dibenzofuran, a carbazole, an acridine, an acridone, a phenanthridine, a thiophene, a benzothiophene, a dibenzothiophene, a xanthene, a xanthone, a flavone, a coumarin, or an anthacyline. Q is either a precursor for producing reactive intermediates such as free radicals, nitrenes, carbenes, and the like, and may be an azide (-N₃), a cyclic azo compound (-N=N-), or a sulfenate (-O-S-).

In one particular embodiment of a therapeutic conjugate, the conjugates have the general formula



wherein Ab is directed at a receptor for steroids, bombesin, neurotensin, or heat sensitive bacterioendotoxin; L is a linker of -HNCONH-, -HNCSNH-, -HNCO-, or -S-(N-succinimido)-(CH₂)_nCONH- and n varies from 1-6; PS is a benzene, a polyfluorobenzene, an anthracene, an anthraquinone, a naphthacenedione, a quinoline, an isoquinoline, an indole, an acridine, an acridone, a phenanthridine, a xanthene, a xanthone, or an anthacyline; and Q is an azide (-N₃) or a sulfenate (-O-S-).

In another particular embodiment of a therapeutic conjugate, the conjugates have the general formula



wherein Ab is directed at a receptor for steroids, bombesin, neurotensin, or heat sensitive bacterioendotoxin; L is a linker of -HNCONH-, -HNCO-, or -S-(N-succinimido)-(CH₂)_nCONH- and n varies from 1 to 6; PS is a tetrafluorobenzene, a phenanthridine, a xanthone, an anthraquinone, an acridine, or an acridone; and Q is an azide (-N₃).

The internal image antibodies can be labeled or coupled with dyes or photosensitizers (i.e., effector molecules) in either a non-selective or selective manner. In non-selective labeling, the photoactive molecule is bound at any location on the anti-idiotypic antibody. This may be accomplished, for example, by using the active esters of the chromophore, fluorophore, or photosensitizer. In selective labeling or coupling, the photoactive molecule is attached distally from the receptor-binding site of the antibody. This may be accomplished, for example, by using a bifunctional maleimido reagent that reacts with a free sulfhydryl group generally found in the fragmented antibodies. Both selective

and non-selective labeling are known to one skilled in the art and have been described by Nicolotti et al. (U.S. Patent No. 4,732,974) and by Achilefu et al. (Investigative Radiology, 2000, 35(8), 479-485), each of which is incorporated by reference herein in its entirety. A typical preparation of a whole or Fab'

5 internal image antibody conjugate bearing a chromophore or a fluorophore is shown in FIG. 1, and that for a photosensitizer is shown in FIG. 2. Both types of preparations are described in detail in the Examples.

Methods of performing diagnostic and/or therapeutic procedures with compositions of the invention are also disclosed. The methods encompass
10 administering to a patient an effective amount of the compositions of the invention contained in a pharmaceutically acceptable formulation.

The compositions are administered in doses or concentrations that are effective to achieve the desired diagnostic or therapeutic objective. Such doses or concentrations may vary widely, depending upon the particular
15 complex employed, the organs or tissues to be examined, the equipment employed in the clinical procedure, and the like. In one embodiment, the dose of the photoactive conjugate may vary from about 0.1 mg/kg body weight to about 500 mg/kg body weight. More typically, the dose is in the range of about 0.5 mg/kg body weight to about 2 mg/kg body weight. In another embodiment,
20 a dose for parenteral administration contains the photoactive conjugate in a concentration ranging from about 1 μ M to about 0.5 M. Preferred parenteral formulations have a concentration in the range of about 1 mM to about 10 mM. The methods for administering different doses and compositions of the conjugates for photodiagnostic and phototherapeutic purposes are known to
25 one skilled in the art.

The inventive compositions can be prepared in diagnostic or therapeutic formulations for enteral (oral or rectal), parenteral, topical or cutaneous administration. Topical or cutaneous delivery may also include aerosol formulations. The compositions may also contain conventional pharmaceutical carriers and excipients appropriate for the type of administration contemplated. Other agents which may be included are stabilizing agents and skin penetration enhancing agents. Such solutions also may contain pharmaceutically acceptable buffers, emulsifiers, surfactants, and electrolytes such as sodium chloride. Acceptable carriers include those that are suitable for injection such as aqueous buffer solutions, e.g., tris(hydroxymethyl)aminomethane and its salts, phosphate, citrate, bicarbonate, and the like, sterile water for injection, physiological saline, and balanced ionic solutions containing chloride and/or bicarbonate salts of normal blood plasma cations such as Ca^{2+} , Na^+ , K^+ and Mg^{2+} . The carriers may contain a chelating agent, e.g., ethylenediaminetetraacetic acid, calcium disodium salt, or other pharmaceutically acceptable chelating agents.

Formulations for enteral administration may vary widely, as is well known in the art. In general, such formulations are liquids, which include an effective amount of the complexes in aqueous solution or suspension. Such enteral composition may optionally include buffers, surfactants, emulsifiers, thixotropic agents, and the like. Compositions for oral administration may also contain flavoring agents and other ingredients for enhancing their organoleptic qualities. Formulations for topical delivery may also contain liquid or semisolid excipients to assist in the penetration or aerosol spray of the photoactive molecule.

After administration, the photoactive conjugate is allowed to accumulate in the region of interest. For photoactivation, the region is illuminated with light having a wavelength in the range of about 300 nm to about 1200 nm, preferably in the range of about 350 nm to about 850 nm. In phototherapeutic procedures the target site is exposed to light sufficient to activate the photosensitizer and/or precursor to induce necrosis or apoptosis of the impaired tissue. Light of low energy is usually needed to activate the photosensitizer and light of high energy is needed to activate the precursor. If the region of interest is, for example a lesion on the skin surface, the region can be directly illuminated. Otherwise, endoscopic catheters equipped with a light source may be employed to provide the photodiagnostic and/or the phototherapeutic effect. The intensity, power, and duration of the illumination, and the wavelength of the light may vary widely depending on the body location, the lesions site, the effect to be achieved, etc. The fluence rate is preferably, but not always, kept below 200 mW/cm² to minimize thermal effects. Appropriate power depends on the size, depth, and the pathology of the lesion, as is known to one skilled in the art. Detection of the chromophore or fluorophore in the localized tissues may be by absorbance, fluorescence, scattering, planar imaging, or tomographic imaging methods known in the art.

The internal image anti-idiotypic antibodies and the corresponding photoactive conjugates can be prepared in accordance with a typical procedure pertaining to digoxin receptor set forth in Example 1, which is not intended to be limiting. Digoxin, like steroids, belongs to a general class of perhydrocyclopentanophenanthrene molecules and binds to the digoxin receptor, which is a special type of myocardial sodium-potassium ATPase. It is

believed that the labeled digoxin internal image antibodies may be useful in the diagnosis of some coronary disorders and/or may supplement the information gained from the use of myocardial perfusion agents.

Example 1

5 **Preparation of digoxin internal image antibody Fab' fragments and conjugation to an optical diagnostic agent.**

- (a) Fusion of mouse myeloma cells with the spleen cells of AJ mice immunized with Balb-C mouse anti-digoxin antibody.

FOR EVIDENCE

Monoclonal antibodies were produced using standard hybridoma

10 technology, as is known to one of ordinary skill in the art. Briefly, two AJ mice were immunized with murine (Balb-C) monoclonal anti-digoxin antibody (Medex Laboratories). A booster injection was given three weeks after the primary immunization and the spleens were removed after three days. Mouse myeloma and spleen cells were washed three times with Dulbecco's Modified Eagle

15 Medium (DME) and suspended in DME (10 ml). A 5 mL portion of each of these cell suspensions was mixed and centrifuged. The supernatant was discarded and the pellet was treated with 1 mL polyethylene glycol (added over a 45 second period), 3 mL DME (added over a 30 second period), and additional 9 mL of DME was added over a 30 second period. The cells were

20 allowed to stand at ambient temperature for eight minutes, and at 37° C for two minutes. The cells were centrifuged, suspended in medium that contains hypoxanthine, aminopterin, and thymidine (HAT medium) (10 mL), and distributed in microtiter plates. The cells were allowed to grow and were screened by a radioimmunoassay procedure approximately 3 weeks after

25 fusion.

(b) Screening of anti-idiotypic antibodies for digoxin.

A 75 μ L portion of affinity purified goat anti-mouse antibody (2 mg/mL) was diluted with phosphate buffered saline (PBS) (150 mL) and 200 μ L aliquots were placed into each well of a microtiter plate. The plates were incubated at 37°C for four hours. The wells were washed with water (three times), treated with 3% BSA solution (200 μ L), and then incubated for one hour. The wells were washed again with water (three times), treated with the supernatants from the cell culture (150 μ L), and then allowed to incubate at ambient temperature for about 18 hours. The wells were washed with water (three times), treated with 125 I labeled goat anti-digoxin (100 μ L) and incubated for four hours. The plates were subsequently washed and the wells were counted for 125 I. A total of 39 positive wells were identified.

The supernatants (100 μ L) from the corresponding positive wells were mixed with 125 I-digoxin (50 μ L) and were placed for one hour in the microtiter plates which were previously coated with about 0.5 μ g of monoclonal mouse anti-digoxin antibody. The plates were washed with water three times and the amount of radioactivity was measured. Inhibition of 125 I-digoxin binding, compared to the control, indicated a positive test for anti-idiotypes. Four positive wells were identified.

(c) Preparation of Fab' fragments of anti-idiotypic digoxin antibody.

Ascites fluid is obtained in the usual manner by injecting hybridoma cells, prepared according to the procedure outlined in the above section (a), into mouse peritoneum. The anti-idiotypic digoxin antibody is purified from the fluid by three successive precipitations with saturated ammonium sulfate in 20

mM phosphate buffer, pH 6.8. Thereafter, the precipitated protein is dialyzed exhaustively against 20 mM phosphate buffer, pH 6.8. The solubilized monoclonal antibody is purified by ion-exchange chromatography (Whatman C-52 column, 0 to 500 mM NaCl gradient in sodium phosphate buffer, pH 6.8).

- 5 The desired fraction containing the anti-idiotypic digoxin antibody is collected and stored in the same buffer at 4°C.

The Fab' fragment is obtained using a modification of the method reported in Parham et al., J. Immunol. Methods, 1982, 53, 133-173. The desired amount of the anti-idiotypic digoxin antibody (absorbance of 1% solution at 280 nm = 14.4) and cysteine-free papain (Worthington, 2 times crystallized) in the ratio of 1:20 is incubated at 37°C using ten times the volume of digestion buffer (100 mM sodium acetate, 3 mM disodium EDTA, pH 5.5) until the reaction is completed (3-16 hours), as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The digestion mixture is diafiltered (Amicon flow cell, PM-10 membrane) at 4°C using 5 mM Tris-HCl buffer, pH 7.2. The anionic Fc fragment removed from the mixture by ion exchange chromatography (Whatman 52 column, 0 to 100 mM NaCl gradient in 5 mM Tris-HCl buffer, pH 7.2). The F(ab')₂ fragment and inactivated papain are contained in the void volume. The F(ab')₂ fragment is further purified from the papain by Sephadex G-100 size exclusion chromatography using 5 mM Tris-HCl buffer, pH 7.2 as the eluant. The desired antibody fragment elutes in the void volume and is characterized by SDS-PAGE. The F(ab')₂ fragments are stored in aliquots at -70°C.

The F(ab')₂ dimer obtained by papain digestion of the whole antibody is further cleaved to the desired Fab' using thiol reagents such as cysteine or

β_2 -mercaptoethanol. The $F(ab')_2$ incubated at room temperature in the presence of either 10 mM cysteine or β_2 -mercaptoethanol until the reaction is complete (one to four hours). The reaction is stopped with the addition of 30 mM iodoacetamide. as determined by SDS-PAGE. Excess reducing agent, 5 iodoacetamide, and other low molecular weight fragments is removed by size exclusion Sephadex G-25 chromatography using phosphate buffered saline, pH 7.4, (PBS), as the eluant. The Fab' fragment thus obtained is used as soon as possible in order to prevent oxidation of the sulfhydryl groups.

(d) Conjugation of Fab' fragments with an optical diagnostic agent.

10 The conjugation procedure is carried out in a manner similar to that for anti-CEA antibody, as reported in Achilefu et al., Investigative Radiology, 2000, 35, 479-485, which is expressly incorporated herein by reference in its entirety.

With reference to FIG. 1, a mixture of the Fab' fragment (2) and 15 about 20 fold molar excess of the dye active ester (1) is incubated in PBS for four hours. The photodiagnostic conjugate (3) is then purified by Sephadex G-25 size exclusion chromatography using PBS as the eluent, and is subsequently lyophilized.

Example 2

20 Preparation of digoxin internal image antibody Fab' fragments and conjugation to a phototherapeutic agent.

The Fab' fragments (2) obtained in Example 1, step (c) are used for conjugation to a phototherapeutic agent. The conjugation procedure is carried out in a manner similar to the one published previously for human serum

albumin (Achilefu et al., Investigative Radiology, 2000, 35, 479-485), which is expressly incorporated by reference herein in its entirety.

With reference to FIG. 2, a mixture of the Fab' fragment (2) and about 20 fold molar excess of the photosensitizer active ester (4), is incubated in PBS for four hours. The phototherapeutic conjugate (5) is purified by Sephadex G-25 size exclusion chromatography using PBS as the eluant, and is subsequently lyophilized.

Example 3

Preparation of whole digoxin internal image antibodies and conjugation to an optical diagnostic agent.

Whole digoxin internal image antibodies obtained in Example 1, steps (a) and (b) were used for conjugation to a photodiagnostic agent. The conjugation procedure was carried out in a manner similar to the one published previously for human serum albumin (Pandurangi et al., Journal of Organic Chemistry, 1998, 63, 9019-9030).

Example 4

Whole digoxin internal image antibodies obtained in Example 1, steps (a) and (b) were used for conjugation to a phototherapeutic agent. The conjugation procedure was carried out in a manner similar to the one published previously for human serum albumin (Pandurangi et al., Journal of Organic Chemistry, 1998, 63, 9019-9030).

It should be understood that the embodiments of the present invention shown and described in the specification are only specific embodiments of the inventors who are skilled in the art and are not limiting in

any way. Therefore, various changes, modifications or alterations to those embodiments may be made or resorted to without departing from the spirit of the invention and the scope of the following claims. In addition, each of the references previously disclosed is expressly incorporated by reference herein in its entirety.

What is claimed is:

FOR FURTHER INFORMATION